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Antibodies against distinct nuclear matrix proteins are characteristic for mixed connective tissue disease

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SUMMARY

Specific nuclear proteins, separated according to their molecular weight (mol. wt) by polyacrylamide gel electrophoresis (PAGE) and subsequently transferred to nitrocellulose sheets, are able to bind antibodies in sera from patients suffering from different types of connective tissue diseases. Antibodies against a characteristic set of nuclear protein antigens are found in sera from patients with mixed connective tissue disease (MCTD). Screening of 21 MCTD sera revealed a typical immunoblot pattern with major protein antigens of mol. wt 70,000 (20/21) (not identical with the Scl-70 antigen characteristic for scleroderma), mol. wt 31,000 (17/21), two proteins around mol. wt 23,000 (15/21) and two around mol. wt 19,000 (10/21). The 70,000, 23,000 and 19,000 antigens appeared to be rather insoluble nuclear proteins (i.e. components of the nuclear matrix). On behalf of their structural character they were present in nuclei from several types of cells but only in low amounts detectable in salt extracts of thymus acetone powder. The presence of antibodies directed against the mol. wt 70,000 antigen correlated strongly with the diagnosis of MCTD. This 70,000 antigen is not identical with the RNP antigen, a soluble ribonuclease sensitive ribonucleoprotein, since antibodies against nuclear RNP can be separated from anti-nuclear matrix antibodies by affinity chromatography using immobilized thymus salt extract. The distinct character of soluble nuclear RNP and structural nuclear matrix antigens is further supported by the fact that from 14 other anti-RNP sera obtained from patients with systemic lupus erythematosus (SLE), only three contained antibodies against the mol. wt 70,000 protein. Since the immunoblot pattern obtained with MCTD sera mostly was clearly distinguishable from the patterns obtained with sera from patients with related connective tissue diseases our results suggest that the immunoblotting technique might be useful as a diagnostic tool and support the concept of MCTD as a distinct entity.

Keywords mixed connective tissue disease nuclear matrix immunoblotting ribonucleoprotein marker antibody

INTRODUCTION

Mixed connective tissue disease (MCTD) has been described as an apparently distinct rheumatic disease syndrome, characterized by a combination of features similar to those of systemic lupus erythematosus (SLE), systemic sclerosis (scleroderma) and polymyositis. Serologically, MCTD is characterized by sometimes extremely high titres of antibody to ribonucleoprotein (RNP) (Sharp *et*

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al., 1972, 1976). The protein component of this antigenic RNP complex, purified by affinity chromatography, has been described to consist of at least 7 different proteins with molecular weights (mol. wt) ranging from 9,000 to 40,000 (Lerner & Steitz, 1979; Takano, Agris & Sharp, 1980; Lerner *et al.*, 1981; Kinlaw, Dusing-Swartz & Berget, 1982; Matter *et al.*, 1982).

Antigenic activity of RNP as demonstrated by immunodiffusion or counterimmunoelectrophoresis assays, can be destroyed by either ribonuclease (RNAase) or protease (Sharp *et al.*, 1976; White, Gardner & Hoch, 1981), suggesting that the formation of the immunoprecipitate is dependent on the integrity of both ribonucleic acid (RNA) and protein, although the naked protein contains at least some antigenic sites (White & Hoch, 1981). RNP is further characterized as a set of soluble ribonucleoproteins, since it is present in salt extracts of (thymus) nuclei (Kurata & Tan, 1976).

Recently it was shown by immunofluorescence that sera from MCTD patients also contain antibodies against other nuclear proteins which are components of a rather insoluble deoxyribonuclease (DNAase), RNAase and high salt resistant proteinaceous structure, generally referred to as nuclear matrix (Salden *et al.*, 1982). To investigate whether the occurrence of these antibodies is a more general phenomenon, the antibody composition of various patient sera was investigated using the immunoblotting technique (Towbin, Stachelin & Gordon, 1979). This technique seems to be very useful because of its capacity to demonstrate antigenic activity in a complex mixture of cellular components and, at the same time, providing information about the exact number and size of molecules that contain antigenic target sites. This latter aspect is a direct improvement when compared to affinity chromatography, in which no distinction can be made between molecules containing antigenic sites and components only associated with, or complexed to, antigens. In this study the immunoblotting technique is used as a routine assay to screen sera from patients with MCTD and other connective tissue diseases for the presence of antibodies against nuclear proteins.

MATERIALS AND METHODS

Patients. Sera from 102 patients with anti-nuclear antibodies (ANA) were used in this study. Most patients were seen in the Departments of Rheumatic Diseases of the University Hospital of Nijmegen and the St Maartens-kliniek in Nijmegen. In addition, 16 sera of SLE and MCTD patients with anti-RNP antibodies were obtained from the University Hospital of Groningen and from the Central Laboratory of the Blood Transfusion Service in Amsterdam, nine sera of patients with Sjögren's syndrome from the University Hospital of Leuven (Belgium), three sera of SLE patients with anti-Sm antibodies from the University of Montpellier (France), two anti-Scl-70 sera of patients with systemic sclerosis from the Royal National Hospital, Bath (England) and serum of one patient with MCTD from the Hospital Ziekenzorg, Enschede.

Patients with MCTD had clinical features of SLE, systemic sclerosis and polymyositis and anti-RNP antibodies (Sharp *et al.*, 1972), patients with SLE satisfied the preliminary American Rheumatism Association criteria for this disease and did not have features diagnostic of other connective tissue disorders (Cohen *et al.*, 1971). Patients with systemic sclerosis satisfied the preliminary criteria for systemic sclerosis (Masi & Rodnan, 1981). Patients with polymyositis had proximal muscular weakness, elevated muscle enzymes, abnormal muscle biopsy and electromyogram (Bohan *et al.*, 1977). Patients with Sjögren's syndrome had keratoconjunctivitis sicca or salivary gland abnormalities with anti-SS-B antibodies. Rheumatoid arthritis patients had classical or definite disease (Ropes *et al.*, 1958).

All sera showed a positive nuclear immunofluorescent staining pattern when acetone fixed cryosections of rat liver tissue and monolayers of cultured cells were used as substrate. Clinical and serological characteristics of the 21 MCTD patients are presented in Table 1. All 21 sera from patients suffering from MCTD showed a speckled nuclear immunofluorescent pattern with nucleoli negative and antibodies to RNP when tested by counterimmunoelectrophoresis (CIE) using a saline extract of rabbit thymus powder (Pel Freez, Rogers, Arkansas) generally referred to as extractable nuclear antigen (ENA) as antigen source (Kurata & Tan, 1976), two of 21 showed anti-DNA antibodies in the Crithidia assay (Aarden *et al.*, 1975), nine of 21 rheumatoid factor using the

Table 1. Clinical and serological characteristics of 21 patients with MCTD

Characteristics	%	
Polyarthralgia/polyarthritis	100	
Raynaud's phenomenon	90	
Myalgia/myopathy	62	
Swollen hands	55	
Fever	43	
Decreased pulmonary diffusion capacity	41	(17)*
Myositis (elevated CPK, abnormal EMG or muscle biopsy)	33	
Skin rash (e.g. butterfly, sunlight sensitivity)	29	
Decreased oesophageal motility	28	(18)
Sclerodermatous changes	19	
Lymphadenopathy/splenomegalie	19	
Sicca syndrome	10	
Serositis	10	
Neurological abnormalities	5	
Renal disease	5	
Laboratory features		
Positive for ANA, speckled pattern	100	
Positive for RNP (CIE)	100	
Positive for (U_1)RNP precipitation	100	
Positive for anti-dsDNA (crithidia assay)	10	
Hypocomplementemia	19	
Positive for rheumatoid factor (Waaler–Rose, Latex)	43	

* Figures in parentheses denote No. of patients tested.

Waaler–Rose and Latex fixation tests (Cats & Klein, 1970) (Table 1). Three out of 36 sera from SLE patients were found to contain exclusively anti-Sm antibodies in CIE whereas 14 of 36 SLE sera contained anti-RNP antibodies. Two of these contained weak anti-Sm activity as well. Sera were, when not immediately used, stored in liquid N_2.

Reference sera. Reference sera were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, and from the Division of Rheumatic Diseases, University of Colorado Health Sciences Center, Denver, USA, and used in the CIE assay. Blotting data of these sera were not used since diagnostic parameters were not available.

Cells. HeLa S3 cells were maintained in suspension culture as described (van Eekelen & van Venrooij, 1981). They were shown not to contain Epstein–Barr virus and regularly performed mycoplasma tests were always negative.

Preparation of HeLa cell total nuclear protein fraction. All procedures were carried out at 0–4°C unless stated otherwise.

Cells were harvested on frozen NKM (130 mM NaCl, 5 mM KCl, 1·5 mM $MgAc_2$), pelleted by centrifugation (5 min at 800*g*), washed once with NKM and pelleted again. The cells were resuspended in cold buffer 1 (10 mM NaCl, 10 mM Tris-HCl pH 7·4, 1·5 mM $MgCl_2$) containing 0·5 mM PMSC (phenyl methyl sulphonyl chloride) to inhibit endogenous proteolytic activities. After addition of a mixture of sodium deoxycholate (DOC) and Tween 40 (0·5% and 1%, respectively), the cells were homogenized by 10 strokes of a motor driven teflon pestle in a Potter–Elvehjem type tissue homogenizer. The nuclei were pelleted (5 min at 800*g*), washed once with buffer 1 containing 0·5 mM PMSC and resuspended in buffer 2 (110 mM NaCl, 10 mM Tris-HCl pH 7·4, 1·5 mM $MgCl_2$,

0·5 mM PMSC) at a density of 10^8 nuclei/ml. To degrade nucleic acids, the nuclei were incubated with 500 μg/ml DNAase 1 and 100 μg/ml RNAase A for 1 h at 20°C. These nuclease treated nuclei are referred to as total nuclear protein fraction.

Preparation of HeLa cell nuclear matrices. HeLa cell nuclei were treated with DNAase 1 (500 μg/ml) for 1 h at 20°C. Chromatin fragments and RNP complexes were removed by sedimentation through a 1 M sucrose layer in buffer 2 followed by an extraction with 0·4 M $(NH_4)_2SO_4$ (van Eekelen & van Venrooij, 1981). The matrices were resuspended in buffer 1, treated with RNAase (100 μg/ml) for 30 min at 20°C and dissolved completely in sample buffer for gel electrophoresis (see below). Morphological and biochemical characteristics of nuclear matrices isolated in this way have been described (van Eekelen *et al.*, 1982).

Gel electrophoresis and protein blotting. Samples for polyacrylamide gel electrophoresis (PAGE) in sodium dodecylsulphate (SDS) were prepared by dissolving the protein fraction in sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0·1 M Tris-HCl pH 6·8). To ensure complete dissociation of the protein complexes, the samples were heated for 3 min at 100°C followed by gel electrophoresis in SDS on 13% polyacrylamide slab gels (1 mm thick, 10 × 16 cm). Proteins were loaded over the entire width of the gel (1·5 mg of protein per gel) and separated according to their molecular weight essentially as described by Laemmli (1970). After a 4 h run at 20 mA per gel, replicas of the gels were made on nitrocellulose (i.e. blotting) by transferring the proteins electrophoretically using a Bio-Rad trans-blot cell. Transfer was performed overnight at room temperature and at 60 V/0·3 A in 192 mM glycine, 25 mM Tris pH 8·3 and 20% methanol. After transfer the blots were dried and stored at room temperature.

Detection of antigens. The protein blots were cut from top to bottom into strips of about 7 mm and treated with pre-incubation buffer (3% bovine serum albumin [BSA], 350 mM NaCl, 10 mM Tris-HCl pH 7·6, 0·5 mM PMSC) for 3 h at 20°C to saturate additional protein binding sites on the nitrocellulose. Incubation with diluted serum (mostly 1:50) was performed overnight in buffer 3 (0·3% BSA, 150 mM NaCl, 10 mM Tris-HCl pH 7·6, 0·1 mM PMSC, 1% Triton X-100, 0·5% DOC and 0·1% SDS). After extensive washing with buffer 3 (3 × 10 min) IgG immunecomplexes were detected by incubating the blots for 2 h with ^{125}I-labelled protein A (spec. act. 1 mCi/mg) in buffer 3 (2 μCi in 20 ml) then washed again with buffer 3 (3 × 10 min) and water (3 × 10 min), dried under a lamp and exposed to X-ray film for 2–16 h at −70°C using Ilford intensifying screens.

In some blotting experiments ^{125}I-labelled anti-human IgG or horse radish peroxidase conjugated anti-human IgG was used instead of protein A. The results were the same.

Absorption of anti-nuclear RNP antibodies. Twenty milligrams of a saline extract of rabbit thymus powder (ENA) which has been shown to contain the soluble RNP antigens (Kurata & Tan, 1976) was immobilized on 3 g (wet weight) of cyanogen bromide activated Sepharose (Pharmacia). Antibodies against nuclear RNP were absorbed by repeated incubation of 1 ml 1:10 diluted serum in BTP (0·5% BSA, 0·5% Triton X-100, in 10 mM PBS, pH 7·5) with 3 ml ENA-Sepharose for 5 h at 20°C under continuous shaking. The supernatant was tested for remaining anti-nuclear RNP activity by counterimmunoelectrophoresis (CIE), ELISA and immunoblotting. The retained anti-RNP antibodies were eluted from the affinity column with 1 M acetic acid and subsequently neutralized with 1 M Tris.

RESULTS

Sera from 35 patients with anti-RNP antibodies (21 MCTD patients and 14 SLE patients) and 67 other ANA positive sera (see Materials and Methods) were tested for the presence of antibodies against nuclear proteins using the immunoblotting technique.

The immunoblotting assay

The assay was routinely performed as described in the Methods section using as source of antigens the nuclear proteins of HeLa S3 cells. Special care was taken to ensure that all DNA and RNA was degraded completely by the incubation with DNAase I and RNAase A. Control experiments (van Eekelen *et al.*, 1982) showed that about 0·5% of the DNA and less than 5% of the nuclear RNA

resisted the nuclease treatment probably as a result of protection by proteins. This heterogeneous mixture of nucleic acid fragments, however, is dissociated from their protecting proteins by the subsequent boiling in SDS. Therefore, under the electrophoretical conditions used in our assay these DNA and RNA fragments are in no way able to contribute to the distinct pattern of protein bands found (Fig. 1, lanes a and b). To prove the proteinaceous character of the antigens more unequivocally, the nuclear protein fraction was, after nuclease treatment, incubated with pronase. After gel electrophoresis and blotting of this mixture, no antigenic activity was detectable anymore (not shown). After electrophoretic transfer of the proteins, the nitrocellulose sheet can be used for the detection of antigens (Fig. 1, lanes c and d). The binding of proteins to nitrocellulose was always sufficient for recognition by antibodies. Binding of antibodies to slowly migrating high molecular weight proteins as large as mol. wt 120,000 could easily be detected when sera in a dilution of 1:50 were used.

When the total nuclear protein fraction was blotted and this blot was incubated with an MCTD serum a pattern of antigens as shown in Fig. 1c was obtained. As described in the Materials and Methods section, the total nuclear fraction can be subdivided in a nuclear matrix fraction and an $(NH_4)_2SO_4$–sucrose fraction. This lattern fraction contains most if not all of the chromatin and high

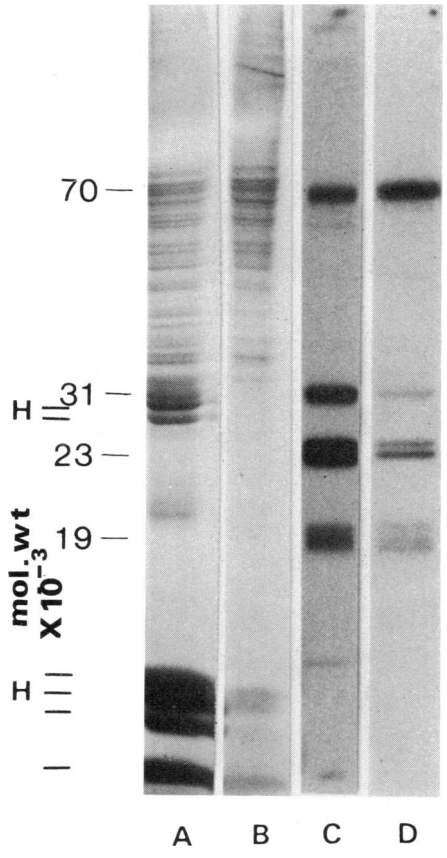

Fig. 1. Detection of nuclear and nuclear matrix antigens with an MCTD serum. HeLa S3 nuclear fractions, treated with DNAase I and RNAase A as described in the Materials and Methods section were completely dissolved in SDS containing sample buffer, heated for 3 min at 100°C and subjected to SDS-PAGE. Electrophoretic transfer of the proteins to nitrocellulose sheets and detection of the antigens was performed as described in the Materials and Methods section. (A) Coomassie brilliant blue stained pattern of total nuclear proteins of HeLa S3 cells; H = Histones. (B) Coomassie brilliant blue stained pattern of nuclear matrix proteins. (C) Immunodetection of nuclear antigens after blotting of lane A and incubation with MCTD serum No. 19. (D) Immunodetection of nuclear matrix antigens after blotting of lane B and incubation with MCTD serum No. 19.

salt extractable RNP structures while the matrix fraction contains matrix components and matrix associated proteins resisting the high salt wash (van Eekelen *et al.*, 1982). The nuclear antigens recognized by the MCTD serum are predominantly present in the matrix fraction (lane d) and have mol. wts of 70,000, 23,000 and 19,000, respectively.

The main advantages using the protein blotting technique for the detection of antibodies are its sensitivity and remarkable reproducibility. Serum from a patient used as a reference for a period of more than 1 year always showed the same characteristic immunoblot pattern, independent from the fact that nuclear protein fractions were used from various batches of HeLa S3 cells. It is by virtue of this reproducibility that we became aware of the characteristic set of antigens recognized by antibodies in sera from patients suffering from MCTD.

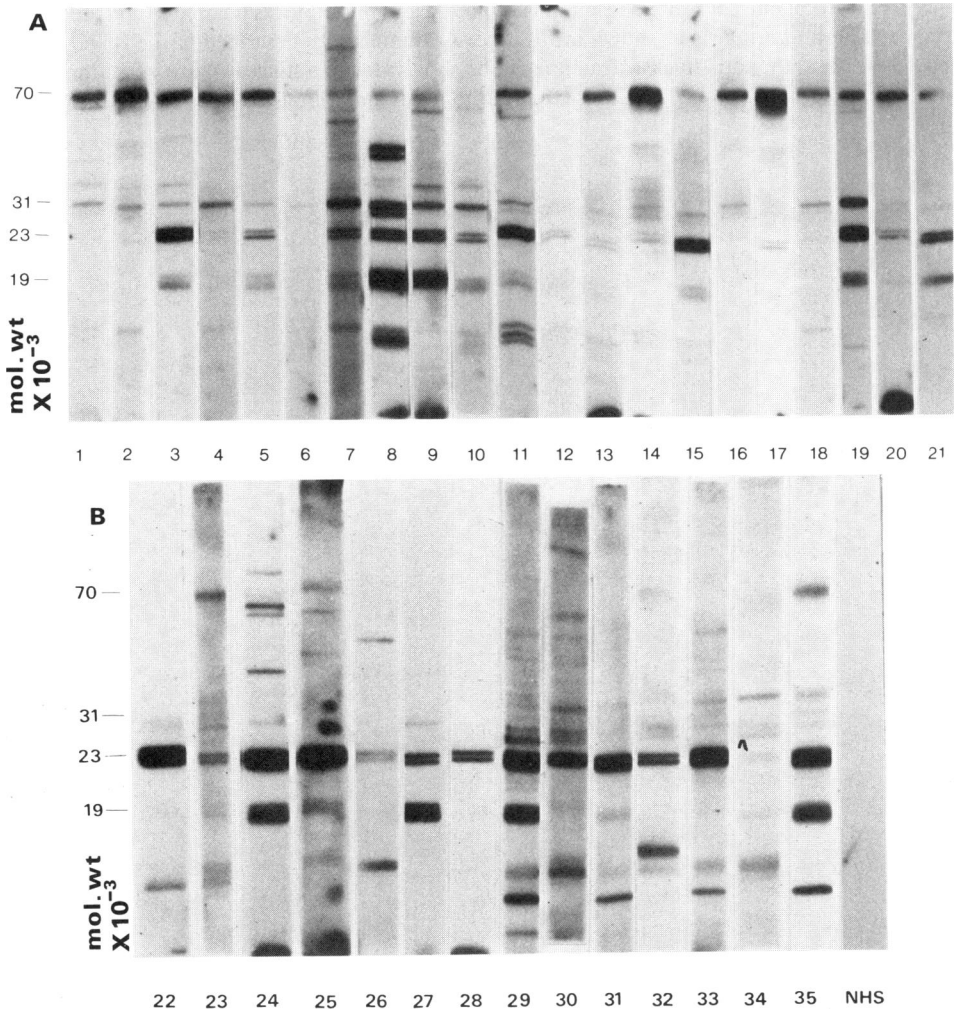

Fig. 2. Immunoblot patterns of patients sera. (A) MCTD sera. Protein blots of a total nuclear protein fraction from HeLa cells were pepared as described in the Methods section. One entire protein blot was cut into strips and each strip was incubated with serum of a patient suffering from MCTD (clinical and serological parameters of these patients are presented in Table 1). Detection of IgG–antigen complexes was performed by incubation with ^{125}I-labelled protein A. (B) Anti-RNP containing SLE sera. Protein blots were incubated with anti-RNP sera obtained from 14 SLE patients (see Methods section). NHS: protein blot incubated with normal human serum obtained from a pool of 40 healthy individuals.

Correlation between MCTD diagnosis and presence of specific antibodies

Comparing the protein blot patterns of sera from MCTD patients (Fig.2A) and SLE sera containing anti-RNP activity (Fig. 2B) it was striking that the presence of the mol. wt 70,000 band correlated strongly with the diagnosis of MCTD (Table 2). As is shown in Fig. 2, 95% of the MCTD sera (20 of 21) recognized the mol. wt 70,000 protein, whereas of the remaining 14 anti-RNP sera, all from SLE patients, only three (21%) did recognize the mol. wt 70,000 antigen. Other commonly found target antigens for antibodies in the MCTD and SLE anti-RNP sera tested were a doublet at mol. wt 23,000 (80%, 28 of 35), two proteins around mol. wt 19,000 (43%, 15 of 35) and a single protein with mol. wt 31,000 (69%, 24 of 35). The presence of multiple other (low molecular weight) antigen bands is particularly evident in the SLE sera. Antibodies against the mol. wt 23,000 doublet, probably identical to the C proteins of Lerner *et al.* (1979) were also found in anti-Sm sera (three of three, Table 2).

Table 2. Occurrence of antibodies against MCTD associated antigens in various sera

Diagnosis	Number of patients	Molecular weight $\times 10^{-3}$ 70	31	23	19
MCTD	21	20 (95%)	17 (81%)	15 (71%)	10 (48%)
SLE anti-RNP	14†	3 (21%)	7 (50%)	13 (93%)	5 (36%)
anti-Sm	3	0	2 (67%)	3 (100%)	0
other	19	1 (5%)	6 (32%)	7 (37%)	1 (5%)
all	36	4 (11%)	15 (42%)	23 (64%)	6 (17%)
PM*	8	0	3 (38%)	1 (13%)	1 (13%)
RA	10	0	0	0	0
PSS	14	0	0	3 (21%)	0
SS	13	0	1 (8%)	0	0

* PM = polymyositis; RA = rheumatoid arthritis; PSS = progressive systemic sclerosis; SS = Sjögren's syndrome..

† Two of these sera contained some anti-Sm activity as well.

Other sera

Although the majority of other investigated ANA positive sera (including sera from patients suffering from Sjögren's syndrome, $n=13$; progressive systemic sclerosis, $n=14$; systemic lupus erythematosus, $n=22$; polymyositis, $n=8$ and rheumatic arthritis, $n=10$) contained antibodies against distinct nuclear proteins (data not shown), the pattern of the target antigens of these sera (number and molecular weights of antigen bands) always was clearly distinguishable from the MCTD type pattern shown in Fig. 2A (see also Table 2).

Presence of the MCTD associated antigens in other cell types

An important and often neglected point in the discussion about the character of nuclear antigens has been the nature of the antigen preparation used in various studies. The fact that antigens might be present both in a soluble (Kurata & Tan, 1976; Sharp *et al.*, 1976) and an insoluble form (White, Billings & Hoch, 1982; Salden *et al.*, 1982) has prompted us to use in our blotting assay the total nuclear protein fraction containing structural as well as soluble components of the HeLa cell nucleus. In Fig. 1 we have shown that the insoluble nuclear matrix fraction, after dissolving it in SDS containing buffer, contained the mol. wt 70,000, 23,000 and 19,000 proteins as the major antigens when an MCTD serum was used. Since these structural proteins constitute only a small fraction of the total cellular protein and thereby are rather insoluble in physiological salt solutions, it is not unexpected that the concentration of these antigens in the commonly used salt extracts of thymus powder is very low as compared with total thymus powder (Fig. 3, lanes F and G). Better

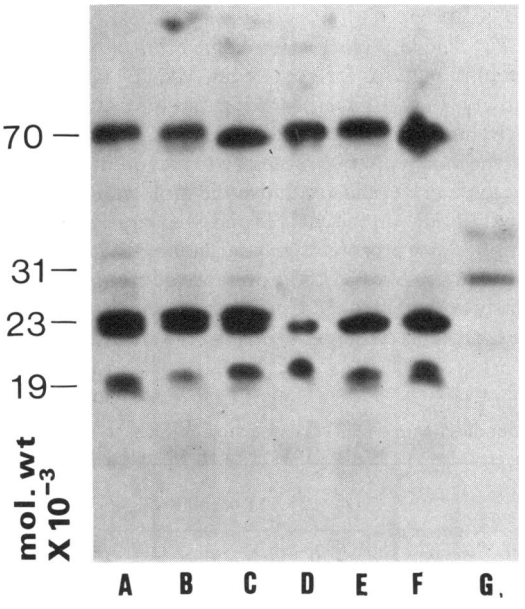

Fig. 3. Occurrence of MCTD associated antigens in various cell lines and tissues. Purified nuclei from (A) HeLa S3 cells, (B) human lymphoblasts in culture (Rosenfeld *et al.*, 1977), (C) cultured hepatoma cells (Alexander *et al.*, 1976) and (E) hamster lens cells (Bloemendal *et al.*, 1980) were prepared as described for HeLa cells in the Methods section. Nuclei from rat liver tissue (D) were prepared as described (Blobel & Potter, 1966). The nuclear protein fractions from these nuclei were prepared by DNAase I and RNAase A digestion followed by SDS solubilization as described in the Methods section. Total rabbit thymus powder (F) was sonicated prior to DNAase I and RNAase A digestion and SDS solubilization. Salt extractable nuclear proteins (G) were isolated from rabbit thymus powder using the procedure of Kurata & Tan (1976). Equivalent amounts of all protein samples were used in the protein blotting assay. All strips were incubated with serum from MCTD patient No. 3 and after ^{125}I-labelled protein A treatment autoradiographed for 16 h.

sources of these antigens, therefore, are purified nuclei from either cultured cells or tissues. Fig. 3, lanes A to E, show the antigens recognized by an MCTD serum in nuclear proteins from various types of cultured cells and rat liver tissue. It is interesting to note here that, for example, in HeLa and lymphocyte nuclear proteins there is a double mol.wt 23,000 antigen band, whereas in rat liver and hamster lens nuclear proteins this antigen appears as a single polypeptide. This phenomenon might be caused by post-synthetic modification of the 23,000 protein which occurs in one type of cell but not in another.

MCTD-70 is not identical with Scl-70

Because of the similarity in molecular weight it was investigated whether the MCTD 70,000 antigen could be identical with the Scl-70 antigen that is often found in sera from patients with progressive systemic sclerosis (Douvas, Achten & Tan, 1979). When Scl-70 sera, which had been shown to react positively in immunodiffusion and immunofluorescence tests (Catoggio *et al.*, 1982) were examined in the protein blotting assay, they did not recognize an antigen of mol. wt 70,000. This strongly suggests that the Scl-70 and the MCTD-70 antigens are not identical. Moreover, none of the 14 sera from scleroderma patients showed antibodies against an mol. wt 70,000 protein in the blotting assay. It is possible that the antibodies against Scl-70 recognize a delicate three-dimensional conformation of the antigen *in situ* that is destroyed during our preparation procedure.

MCTD associated nuclear matrix antigens are distinct from nuclear RNP

The distinct character of nuclear RNP and the MCTD associated nuclear matrix antigens is already indicated by the fact that from 14 anti-RNP sera (all obtained from SLE patients) only three did

recognize the mol. wt 70,000 nuclear matrix antigen. Moreover, none of the matrix antigens were detectable in a saline extract of rabbit thymus (ENA, Fig. 3 lane G). To prove the distinct character of the nuclear matrix antigens more unequivocally the following experiment was performed. An MCTD serum was passed over an ENA-Sepharose column (see Materials and Methods) to remove the antibodies against nuclear RNP. To make sure that the absorption was quantitative, the non-bound antibody fraction was tested in counter-immunoelectrophoresis, by immunoblotting and in a very sensitive ELISA technique, always using ENA as the substrate. No reactivity towards soluble nuclear RNP antigens was detectable anymore (not shown). However, anti-nuclear matrix antibodies were still present in the non-bound antibody fraction as is shown in Fig. 4 lane B. When the retained anti-nuclear RNP antibodies were eluted from the affinity column, they did not show any reactivity towards the nuclear matrix antigens (Fig. 4 lane C) but appeared to be enriched in activity towards polypeptides with mol. wts of 9,000–14,000.

Summarizing we conclude that MCTD sera recognize a specific set of nuclear protein antigens of mol. wt 70,000, 31,000, 23,000 and 19,000. Some of these proteins are also recognized by other anti-RNP sera (Table 2 & Fig. 2B). However, the presence of antibodies against a mol. wt 70,000 antigen, mostly in combination with (some of) the other three antigens is highly characteristic for MCTD.

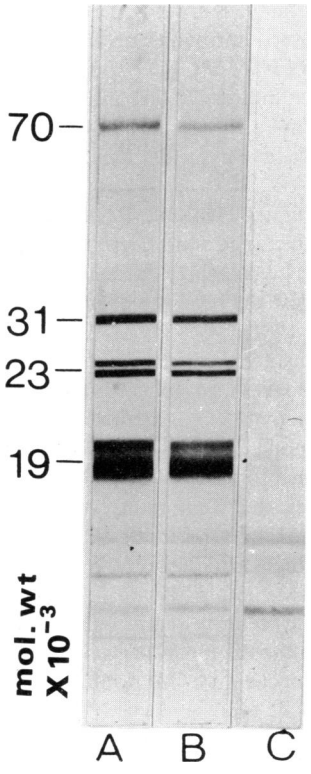

Fig. 4. Detection of separated anti-RNP and anti-nuclear matrix antibodies. Serum from MCTD patient No. 19 was depleted from antibodies against nuclear RNP by absorption on an ENA–Sepharose affinity column. The non-bound fraction and the eluate were tested by immunoblotting. Three identical strips of the same protein blot of a HeLa total nuclear protein fraction were incubated with: (A) Serum before absorption; (B) serum after absorption (non-bound fraction); (C) purified anti-nuclear RNP antibodies (eluate). The strips were incubated for 1 h with horse radish peroxidase conjugated anti-human IgG and peroxidase activity was detected by incubating the washed blots in PBS (10 mM phosphate buffer in 0·9% NaCl, pH 7·5) containing 4-chloro-1-naphtol (0·5 mg/ml) and H_2O_2 (0·06%).

DISCUSSION

The use of the immunoblotting technique for the detection of specific antigens recognized by antibodies in autoimmune sera has several advantages. Because not only the soluble but also the insoluble antigens are dissolved in SDS before gelelectrophoresis, more complete information is obtained about the number and size of the antigens. In contrast to affinity chromatography or immunoprecipitation, in the immunoblotting technique the antigenic complexes are first separated into their individual constituents, so that only the antigenic sites bearing polypeptides are detected. Moreover, the method is extremely sensitive (Towbin *et al.*, 1979) and reproducible as has been pointed out already in the Results section. Finally the blotting technique can be used for the screening of large groups of patient sera.

The group of 21 MCTD patients examined in this study is a rather heterogeneous one as far as their clinical characteristics are concerned (Table 1). All 21 MCTD sera, however, showed a speckled nuclear immunofluorescent pattern, a finding not only characteristic for MCTD sera but also often found when, for example, SLE sera are used. Furthermore, all 21 sera contained precipitating antibodies to (U_1)RNP when tested in counterimmunoelectrophoresis and immunoprecipitation studies (Table 1). The antibodies to RNP have been described as being characteristic for MCTD (Sharp *et al.*, 1972) although they can be found also (in somewhat lower titres and frequency) in sera from patients suffering from SLE, rheumatoid arthritis, Sjögren's syndrome and progressive systemic sclerosis (Notman, Kurata & Tan, 1975). Although all ANA positive sera tested with immunoblotting contained antibodies against a great variety of nuclear proteins, the antigen patterns obtained with sera from MCTD patients were surprisingly similar. In particular the presence of antibodies against a mol. wt 70,000 antigen in almost all MCTD sera was striking and allowed us to distinguish them easily among immunoblot patterns of about 100 sera from patients with various types of connective tissue diseases, even when these sera contained anti-RNP activity (Table 2 & Fig. 2).

It is commonly agreed upon that the soluble RNP complex precipitated by anti-RNP sera contains at least seven proteins in the range of mol. wt 9,000–40,000 (Lerner & Steitz, 1979; Takano *et al.*, 1980; Sri-Widada *et al.*, 1982; Lenk, Maizel & Crouch, 1982; Matter *et al.*, 1982; Kinlaw *et al.*, 1982) although some reports suggest the presence of mol. wt 70,000 and 65,000 proteins as well (White *et al.*, 1982; Takano *et al.*, 1981).

However, these soluble antigens are distinct from the nuclear matrix antigens since we have demonstrated here than an MCTD serum, completely depleted from anti-nuclear RNP antibodies still contains anti-nuclear matrix activity. The fraction containing the antibodies against nuclear RNP showed reactivity towards polypeptides with mol. wts of 9,000–14,000 present in a total (soluble as well as insoluble) nuclear protein fraction from HeLa cells. Antibodies against such low molecular weight proteins in this region are commonly found in anti-RNP sera (White *et al.*, 1981; Sri-Widada *et al.*, 1982; Lenk *et al.*, 1982). It is interesting to note further that these low molecular weight proteins are recognized in a higher frequency by RNP sera obtained from SLE patients (11 of 14) than by the MCTD sera.

In summary we conclude that the presence of antibodies against a mol. wt 70,000 nuclear matrix antigen is strongly correlated with the diagnosis of MCTD (Table 2) suggesting that this disease indeed might be considered as a distinct entity. Our results further demonstrate the immunoblotting technique to be a powerful diagnostic tool.

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